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Independent regulation of skeletal growth by Ihh and IGF signaling

Fanxin Long [a,b,c,*], Kyu-Sang Joeng [a,c], Shouhong Xuan [d], Argiris Efstratiadis [d], Andrew P. McMahon [e]

[a] Department of Medicine, Washington University Medical School, St. Louis, MO 63110, USA
[b] Department of Molecular Biology and Pharmacology, Washington University Medical School, St. Louis, MO 63110, USA
[c] Division of Biology and Biomedical Sciences, Washington University Medical School, St. Louis, MO 63110, USA
[d] Department of Genetics and Development, Columbia University, New York, NY 10032, USA
[e] Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA

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Abstract

The insulin-like growth factors (IGFs) play a major role in regulating the systemic growth of mammals. However, it is unclear to what extent their systemic and/or local functions act in concert with other local growth factors controlling the sizes of individual organs. We have specifically addressed whether growth control of the skeleton by IGFs interacts genetically with that by Indian hedgehog (Ihh), a locally produced growth signal for the endochondral skeleton. Here, we report that disruption of both IGF and Ihh signaling resulted in additive reduction in the size of the embryonic skeleton. Thus, IGF and Ihh signaling appear to control the growth of the skeleton in parallel pathways.

Keywords: Ihh; Smo; IGF; IGF1R; Growth; Skeleton; Mouse

1. Introduction

The mechanisms controlling the body size of mammals are not well understood. The growth of a mammal occurs throughout embryogenesis and continues postnatally to reach a steady state (Efstratiadis, 1998). The insulin-like growth factors (IGF1 and IGF2) are major growth-promoting signals for both embryonic and postnatal development. Whereas IGF2 is essential for normal embryonic growth (DeChiara et al., 1990, 1991), IGF1 appears to control body size throughout development (Baker et al., 1993; Liu et al., 1993). In regulating postnatal growth, IGF1 functions both as an effector for growth hormone (GH) as well as by a GH-independent mechanism (Lupu et al., 2001). Overall, genetic evidence supports the conclusion that a GH-IGF signaling system plays a predominant role in determining body size in mammals.

Both circulating and locally produced IGF1 may contribute to growth regulation. The original "somatomedin hypothesis" posited that GH stimulates the hepatic production of circulating IGF1 which in turn affects local tissues (Salmon and Daughaday, 1957), but more recent evidence indicates that locally produced IGF1 may exert paracrine or autocrine functions on target tissues. In support of the endocrine role of IGF1, reduction of the circulating IGF1 level to 10–15% of normal by removal of the acid labile subunit (ALS) (which helps to stabilize serum IGF1 levels) along with ablation of *Igf1* expression in the liver resulted in significant growth retardation in postnatal mice (Yakar et al., 2002), even though the liver-specific deletion of *Igf1* alone (resulting in serum IGF1 levels ~25% of normal) did not significantly affect growth (Sjogren et al., 1999; Yakar et al., 1999).

IGF1 and IGF2 are known to signal through the type I IGF receptor (IGF1R) in target tissues, as demonstrated by genetic studies. Deletion of *Igf1r* ($Igf1r^{n/n}$) resulted in lethality at birth, as well as more severe growth deficiency (45% of normal weight) than that caused by *Igf1* or *Igf2* deletion (60% of normal weight) (Baker et al., 1993; Liu et al., 1993). Importantly, $Igf1^{n/n}$; $Igf1r^{n/n}$ double-mutant mice phenocopied the growth defect of $Igf1r^{n/n}$ animals, whereas deletion of both *Igf2* and *Igf1r* caused a more severe growth deficiency than

* Corresponding author. Campus Box 8301, Washington University Medical School, 660 S. Euclid Ave., St. Louis, MO 63110, USA.
E-mail address: flong@wustl.edu (F. Long).

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removal of *Igf1r* alone (30% versus 45% of normal weight) (Liu et al., 1993). Thus, IGF1R mediates all activities of IGF1, as well as a major portion of IGF2 signaling.

Consistent with its role as a systemic growth regulator, IGF signaling has been implicated in skeletal growth. IGF1R is expressed in both chondrocytes and osteoblasts of the developing skeleton (Wang et al., 1995). The $Igf1r^{n/n}$ embryos exhibited a smaller skeleton associated with significant delays in ossification (Liu et al., 1993). Moreover, postnatal analyses of the surviving $Igf1^{n/n}$ mice revealed a reduction in the size of hypertrophic chondrocytes (Lupu et al., 2001; Wang et al., 1999). However, the relationship between IGF and other growth regulators in the skeleton is unclear.

Indian hedgehog (Ihh) is a locally produced signal critical for development of the endochondral skeleton. While it is produced by prehypertrophic and early hypertrophic chondrocytes, Ihh signals to both chondrocytes and the overlying perichondrium (St-Jacques et al., 1999; Vortkamp et al., 1996). Mice lacking Ihh ($Ihh^{n/n}$) exhibited a severe growth deficiency in the endochondral skeleton; skeletal elements were only about 20% of their normal size at birth (St-Jacques et al., 1999). Detailed analyses of the $Ihh^{n/n}$ mice revealed profound defects in chondrocyte proliferation and maturation, as well as osteoblast formation, all contributing to the reduction in skeletal size (St-Jacques et al., 1999). Moreover, chondrocyte-specific deletion of *Ihh* recapitulated all skeletal defects as observed in the $Ihh^{n/n}$ mutant (Razzaque et al., 2005). These studies established Ihh as a potent local growth signal for the endochondral skeleton.

The intracellular transduction of all Hh signals requires the activity of a seven-pass transmembrane protein Smoothened (Smo) (Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Zhang et al., 2001). Genetic manipulation of *Smo* in the skeleton further revealed that, whereas Ihh directly controls chondrocyte proliferation and osteoblast differentiation, it appears to regulate chondrocyte maturation via a secondary signal (Long et al., 2001, 2004), most likely the *parathyroid hormone related peptide* (*Pthlh*) (Karp et al., 2000), in agreement with a model proposed from earlier genetic and *in vitro* analyses (Vortkamp et al., 1996).

In the present study, we sought to determine whether IGF signals regulate skeletal growth by modulating Ihh signaling. Our results support the conclusion that the two signaling systems likely function independently in skeletal cells.

2. Materials and methods

2.1. Mouse strains

All mouse strains including $Ihh^{n/wt}$, $Igf1r^{n/wt}$, $Igf1r^{c/c}$, $Smo^{n/wt}$, $Smo^{c/c}$ and *Col2-Cre* are as previously described (Dietrich et al., 2000; Liu et al., 1993; Long et al., 2001; St-Jacques et al., 1999; Zhang et al., 2001). The *Col2-Cre10* line was used in this study (Long et al., 2001). All animals were genotyped by PCR.

2.2. Morphometry

Mutant animals were first identified based on the previously known phenotypes associated with the single mutations. The body weight of mutant versus normal embryos was obtained immediately following dissection. Skin samples were collected from the mutant animals and subsequently genotyped, whereas animals with normal gross morphology were characterized as "wild type" without genotyping. Whole mount skeletons of mouse embryos or newborns were stained according to a protocol based on McLeod (1980). For measurement of skeletal parameters, individual skeletal elements were dissected from the cleared skeleton and measured under a dissecting scope. Assuming that two single mutations cause the shortening of a skeletal element independently by X% and Y% of normal, respectively, it is predicted that the double mutant would exhibit a reduction of $(X+Y)$%.

2.3. BrdU labeling assay

For BrdU labeling experiments, pregnant females were injected with BrdU 2 h prior to sacrifice as previously described (Hu et al., 2005). The limbs were dissected from the embryos, fixed in 10% buffered formalin overnight at room temperature, processed and finally sectioned in paraffin. The sections were stained for BrdU incorporation using a kit (Zymed Laboratories, CA). BrdU labeling index was scored for multiple sections from each cartilage element. The results reported here were from the forelimbs (digit 1 for the $Igf1f^{n/n}$;$Ihh^{n/wt}$, and the *DKO* embryos; digit 2 for the *DCKO* and wild-type littermates; both the proximal end of the ulna and digit 4 for the $Igfr^{n/n}$ and wild-type littermates).

2.4. Glycogen staining

Glycogen accumulation was detected by the periodic acid-Schiff (PAS) reaction as previously described (Wang et al., 1999). Briefly, sections were deparaffinized, rehydrated and oxidized in 0.1% periodic acid and then stained in 0.5% Schiff's solution (Sigma) and counterstained with hematoxylin.

3. Results

3.1. Global removal of Ihh and IGF1R signaling

To determine whether Ihh and IGF signaling intersects in regulating embryonic growth, we attempted to generate compound, homozygous null mutant embryos that were $Ihh^{n/n}$; $Igf1r^{n/n}$ (*DKO*) by mating double heterozygous mice ($Ihh^{n/wt}$; $Igf1r^{n/wt}$). However, in harvesting a total of 236 embryos at E17.5–18.5, we identified only two *DKO* embryos, at a recovery rate of 0.8%, approximately 1/8 of the expected Mendelian ratio (6.25%), even though both types of single null mutants as well as the $Ihh^{n/wt}$; $Igf1r^{n/n}$ embryos were recovered at, or near, expected ratios (Table 1). Moreover, $Ihh^{n/n}$; $Igf1r^{n/wt}$ embryos were recovered at 3%, also significantly below the expected 12.5%. Thus, in the background of $Ihh^{n/n}$, removal of *Igf1r* resulted in embryonic lethality, apparently in a dose-dependent manner. Subsequent analyses at progressively early stages indicated that a majority of the *DKO* embryos died prior to E11.5 (data not shown), but the reasons for the early lethality were not pursued.

The *DKO* mutant embryos exhibited more severe growth retardation than either single mutant. As previously described, at E17.5, the overall size of either $Ihh^{n/n}$ or $Igf1r^{n/n}$ embryos was significantly smaller than that of the normal littermates (Fig. 1A). Interestingly, the *DKO* embryo was further reduced in size (Fig. 1A). In particular, the body weight of the compound mutant decreased to ~22% of the wild-type embryo, compared to 79% and 44% with the $Ihh^{n/n}$ and the $Igf1r^{n/n}$ embryo, respectively (Fig. 1B). Notably, the weight deficit in the *DKO* embryo (78%) was equivalent to the sum of the deficits in the single mutants (77%=21%+56%), suggesting an additive

Table 1
Breeding data ($Igf1r^{n/wt}$; $Ihh^{n/wt}$×$Igf1r^{n/wt}$; $Ihh^{n/wt}$)

Genotype *Igf1r*/*Ihh*	Number of mice (n=236)	Observed frequency (%)	Expected frequency (%)	Phenotype
++/++	168	71.19	6.25	Normal W: 1.025±0.03
++/+−			12.50	
+−/++			12.50	
+−/+−			25.00	
			56.25	
−−/++	19	8.00	6.25	Lacking *Igf1r*
−−/+−	28	12.00	12.50	W: 0.45+0.04 (43.9% of normal)
++/−−	12	5.00	6.25	Lacking *Ihh*
+−/−−	7	3.00	12.50	W:0.81±0.03 (79% of normal)
−−/−−	2	0.85	6.25	Lacking *Ifr*+*Ihh* W: 0.22 (21.5% of normal)

Average weights (W; in g) ±SEM were calculated from subsets of embryos recovered at E17.5–E18.5 (the number for normal embryos and for *Igf1r*, *Ihh* and double nullizygotes were 4, 4, 3 and 1, respectively).

effect of the mutations. Moreover, the *DKO* embryos maintained all morphological features of $Ihh^{n/n}$ embryos, including a severe reduction of the limbs (red arrows, Fig. 1A). Thus, removal of IGF1R signaling in the absence of Ihh resulted in a further decrease in body size and weight.

The exacerbated growth defect in the *DKO* embryo was evident in the skeleton. As previously reported, $Ihh^{n/n}$ mutants exhibit severe defects in the endochondral skeleton. In particular, the limb skeletal elements (long bones) were very short and disproportionally wide and failed to ossify (Fig. 1C). The $Igf1r^{n/n}$ embryo, on the other hand, had long bones proportionately shorter than normal but considerably longer than those in the $Ihh^{n/n}$ mutant (Fig. 1C). Importantly, in the *DKO* embryo, the long bones maintained the same morphology as in the $Ihh^{n/n}$ mutant but were further reduced in size (Fig. 1C). These results therefore revealed non-overlapping roles between Ihh and IGF1R signaling in the regulation of skeletal growth.

We next investigated whether Ihh and IGF1R signaling controlled chondrocyte proliferation in an interdependent manner. Previous studies showed that $Ihh^{n/n}$ embryos had a 50% loss in BrdU labeling index in chondrocytes (St-Jacques et al., 1999), whereas $Igf1r^{-/-}$ fibroblasts in culture showed a significant defect in proliferation (Sell et al., 1994). We specifically assayed for the BrdU labeling index upon removal of *Ihh* in the absence of *Igf1r*. In this setting, ~13.1% of the

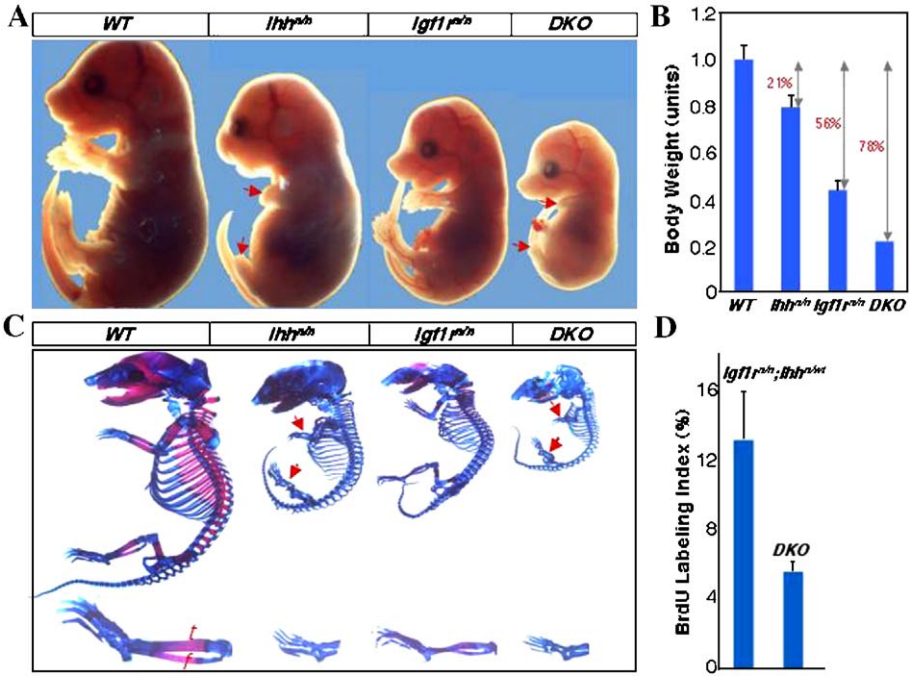

Fig. 1. Analyses of mouse embryos with *Ihh* and/or *Igf1r* removed from all tissues. (A) Gross morphology of E17.5 embryos. Red arrows indicate the diminished limbs in $Ihh^{n/n}$ and *DKO* embryos. (B) Relative body weights of E17.5 embryos. The wild-type weight was designated as 1 unit. n=4, 4, 3 and 1 for wild type, $Igf1r^{n/n}$, $Ihh^{n/n}$ and *DKO*, respectively. (C) Whole mount skeletons at E17.5 stained with alcian blue (cartilage) and alizarin red (bone and mineralized cartilage). Red arrows indicate the diminished limb skeletal elements in $Ihh^{n/n}$ and *DKO* embryos. Hind limbs with the tibia (t) on the top and the fibula (f) at the bottom are shown at a higher magnification below the respective whole mount skeletons. (D) BrdU labeling assays for proliferating chondrocytes in phalanges at E17.5.

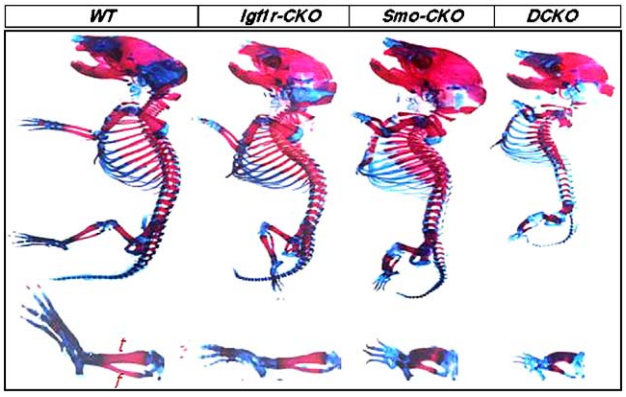

Fig. 2. Skeletons of E18.5 mouse embryos with *Smo* and/or *Igf1r* specifically removed from the cartilage. Hind limbs with the tibia (t) on the top and the fibula (f) at the bottom are shown at a higher magnification below the respective whole mount skeletons.

growth region chondrocytes in the $Igf1r^{n/n}$;$Ihh^{n/wt}$ embryo were labeled by BrdU (Fig. 1D). However, the labeling index dropped to ~5.6% in the *DKO* embryo, indicating a ~57% reduction. Thus, proliferation was decreased to a similar extent when Ihh activity was removed in the presence or absence of IGF1R activity.

3.2. Cartilage-specific removal of Ihh and IGF1R signaling

In order to circumvent premature embryonic lethality and to investigate skeleton-specific effects of Ihh and IGF signaling, we generated mutant mice that were unable to respond to either signal in the cartilage through removal of *Smo*, or *Igf1r*, or both genes specifically in chondrocytes. To this end, we utilized a *Col2-Cre* transgenic line (Long et al., 2001) to delete the floxed alleles of *Smo* (Smo^c) (Long et al., 2001) and *Igf1r* ($Igf1r^c$) (Dietrich et al., 2000). Among a total of 243 animals harvested at birth (P0) from crosses between *Col2-Cre;* $Smo^{n/wt}$; $Igf1r^{n/wt}$, and $Smo^{c/wt}$; $Igf1r^{c/wt}$, five were identified as double conditional knockout mutants (*DCKO*) (2.1%), approximating the expected ratio (~3.1%).

The *DCKO* mice exhibited a further reduction in the skeleton compared with that of either single conditional knockout animal. Specifically, whereas the *Igf1r-CKO* mutant exhibited proportionately shorter elements and the *Smo-CKO* animals contained shortened and misshapen long bones, the limb skeletal elements of the *DCKO* littermates were both misshapen and further reduced (Fig. 2). Thus, as observed with elimination of *Ihh* and *Igf1r* in the entire animal, cartilage-specific removal of the Ihh and IGF responsiveness resulted in a further reduction in the size of the skeleton.

To determine whether the exacerbated phenotype in the *DCKO* animals represented additive or synergistic effects between the single mutations, we measured the linear length of individual skeletal elements and compared them across genotypes. In newborn animals (P0), depending on the individual element, the *Igf1r-CKO* and the *Smo-CKO* mice showed a 10–20% or a 30–50% reduction over the wild type, respectively (Fig. 3). As expected from earlier analyses, the *DCKO* mutants exhibited a further diminution in the length of skeletal elements. Interestingly, the lengths of the *DCKO* elements approximated the theoretical values predicted from independent signaling (see Materials and methods). Thus, disruption of both Ihh and IGF1R signaling in the cartilage appeared to result in an additive defect in skeletal growth.

We next compared the morphology of the growth plate among embryos with the different genotypes. At E18.5, the growth plate of a normal femur was organized in distinct regions containing round cells (“R”), flattened cells (“C”, organized in columns), and hypertrophic cells (“H”) (Fig. 4A). The growth plate in the *Igf1r-CKO* mouse, although shorter and narrower, maintained a general organization similar to that in

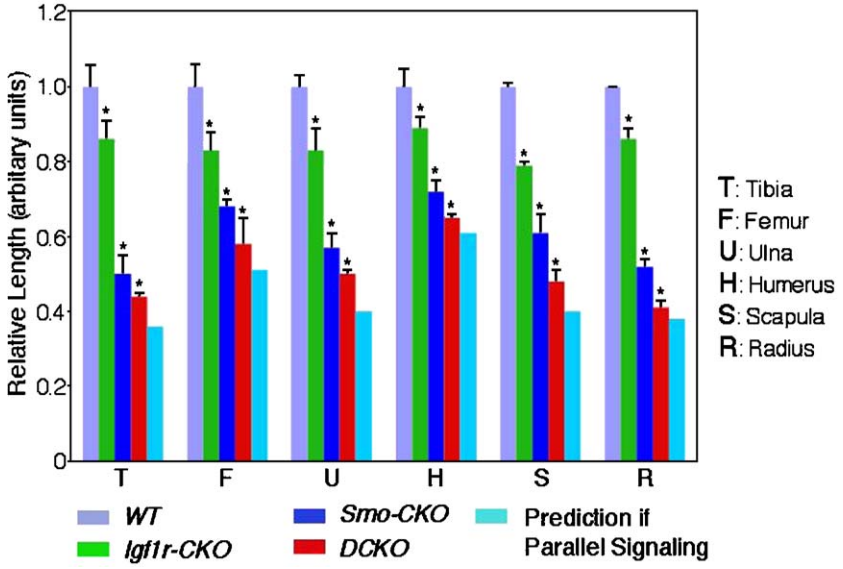

Fig. 3. Relative lengths of skeletal elements in newborn (P0) mice. $N \geq 3$ for all genotypes. The wild type length for each element is designated as 1 unit. Asterisks indicate that there is a statistically significant difference between genotypes in pairwise comparisons ($p < 0.05$). The actual lengths of the *DCKO* elements do not differ significantly from the predicted values based on independent signaling by Ihh and IGF1R.

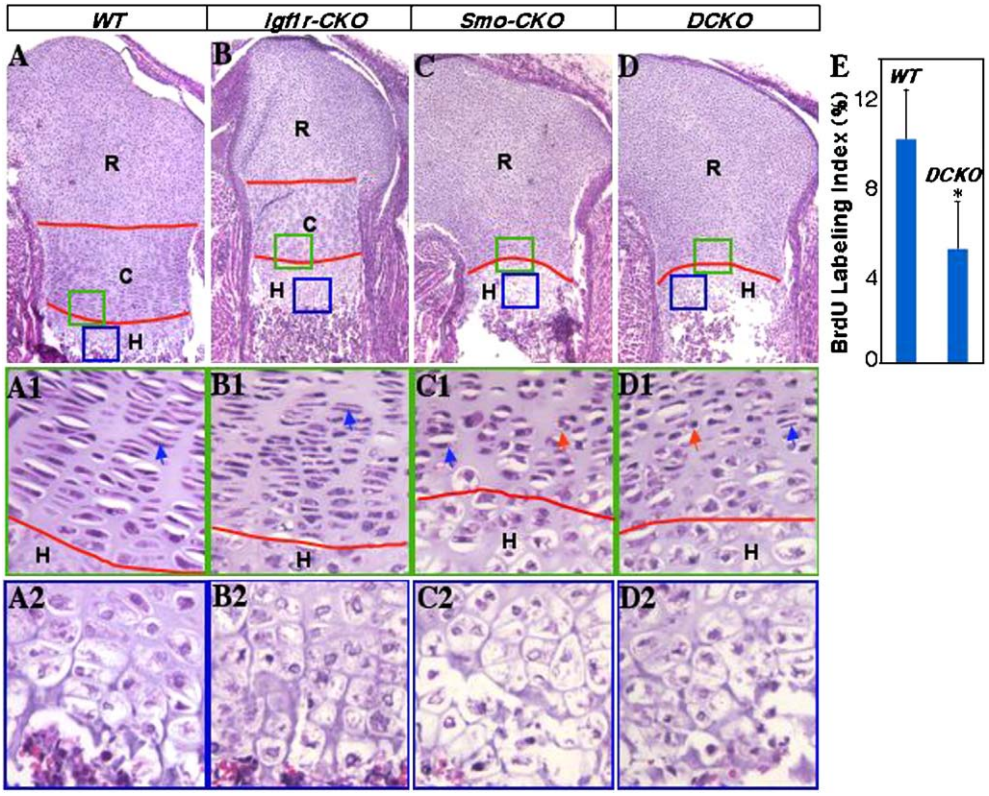

Fig. 4. Growth plate morphology and chondrocyte proliferation at E18.5. (A–D) Histology of the growth plate of the femur in wild-type (A), *Igf1r-CKO* (B), *Smo-CKO* (C) and *DCKO* (D) embryos. (A1–D1) Areas in green boxes in panels A–D, respectively, shown at a higher magnification. (A2–D2) Areas in blue boxes in panels A–D, respectively, shown at a higher magnification. R: round chondrocytes; C: columnar chondrocytes; H: hypertrophic chondrocytes. Blue arrows: flat chondrocytes; red arrows: round chondrocytes. (E) BrdU labeling index in the phalanges of *DCKO* versus wild-type embryos at E18.5. $*p<0.05$.

the wild-type embryo (Fig. 4B). At a higher magnification, the flattened cells (blue arrows) can be seen in stacks in both the wild-type and the *Igf1r-CKO* embryos (Fig. 4, A1 and B1 respectively). However, the size of individual hypertrophic chondrocytes appeared smaller in the *Igf1r-CKO* embryo than in the wild-type littermate (Fig. 4, B2 and A2 respectively), as described previously (Wang et al., 1999). Remarkably, in the *Smo-CKO* mouse, the growth plate had virtually no columnar region, even though the regions for the round cells and the hypertrophic chondrocytes were maintained (Fig. 4C). Instead, the equivalent of the columnar region was now disorganized and contained both round (red arrow) and flattened (blue arrow) cells (Fig. 4, C1). Finally, the growth plate in the *DCKO* embryo was overall similar to that in the *Smo-CKO* littermate, and it too lacked an obvious columnar region (Fig. 4, D and D1). Thus, removal of *Smo* leads to the loss of a distinct columnar region in the growth plate with or without IGF signaling.

To confirm the proliferation defect in chondrocytes lacking both Ihh and IGF signaling, we performed BrdU labeling experiments in *DCKO* and wild-type littermates. At E18.5, the *DCKO* embryos showed a ~50% reduction in the labeling percentage in the phalanges when compared to wild-type controls (Fig. 4E). Interestingly, at the same embryonic stage, the $Igf1r^{n/n}$ embryo showed no reduction in BrdU labeling in either the phalanges or the ulna in comparison with the wild-type level (data not shown; see Discussion). Thus, regardless of the status of IGF signaling, elimination of Ihh signaling significantly reduces the BrdU labeling index in chondrocytes.

Parallel signaling by Ihh and IGF1R would predict normal signaling by Ihh or IGF1R in animals with *Igf1r* or *Ihh* deficiency, respectively. The normal morphology of the growth region cartilage in the $Igf1r^{n/n}$ or the *Igf1r-CKO* mutant (Fig. 5, B1) is consistent with normal Ihh production and signaling. In order to determine whether IGF signaling was affected in chondrocytes in the absence of Ihh, we examined glycogen accumulation as a functional readout for IGF signaling (Wang et al., 1999). At E17.5, abundant glycogen was detected in both proliferating and prehypertrophic chondrocytes of a normal embryo (Fig. 5, A1 and A2), but as expected, this was significantly diminished in the *Igf1r-CKO* mutant (Fig. 5, B1 and B2). Importantly, glycogen levels in the non-hypertrophic chondrocytes of the $Ihh^{n/n}$ embryo were not reduced from wild-type levels (Fig. 5, C1 and C2). As a control, glycogen levels in the skeletal muscle were unchanged across the three genotypes (Fig. 5, A3–C3). These results support the conclusion of functional IGF signaling in the absence of Ihh signaling in chondrocytes.

4. Discussion

We have tested genetically the hypothesis that systemic growth signals may function by modulating signaling by local factors in individual target tissues. In particular, we addressed

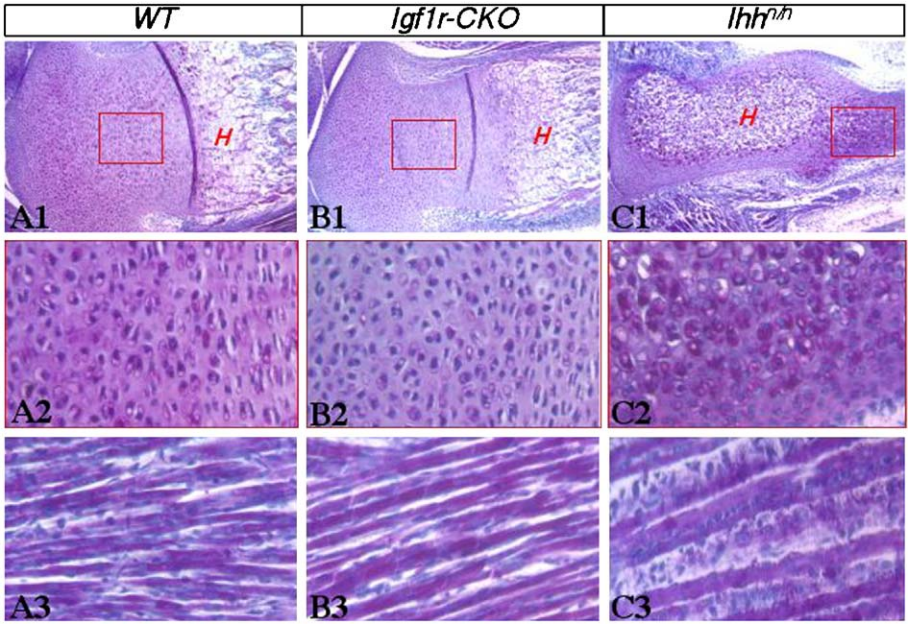

Fig. 5. Glycogen accumulation in growth region chondrocytes. Glycogen was stained in magenta. Boxed regions in (A1–C1) are shown at a higher magnification in (A2–C2), respectively. Skeletal muscles from the same sections are shown as control for the staining procedure (C1–C3). H: hypertrophic chondrocytes.

whether Ihh and IGF signaling might intersect in controlling growth of the skeleton. By disrupting both signaling pathways either globally (*Ihh*$^{n/n}$; *Igf1r*$^{n/n}$) or specifically in the skeleton (*Col2-Cre; Smo*$^{n/c}$; *Igf1r*$^{n/c}$), the evidence suggests that IGFs and Ihh most likely signal independently in the regulation of skeletal growth.

Disruption of either IGF or Ihh signaling pathway resulted in pups developing a smaller skeleton. It should be noted that the two mutant skeletons exhibited apparent differences from each other in their gross morphology. Whereas bones in either *Igf1*$^{n/n}$ or *Igf1r*$^{n/n}$ mice were proportionately smaller than normal, skeletal elements in the *Ihh*$^{n/n}$ mice were more severely reduced and disproportionately wider. The milder phenotype resulting from defective IGF signaling could incorporate a partial disruption of Ihh signaling (IGF upstream of Ihh), or Ihh could regulate skeletal growth in part by controlling IGF signaling (Ihh upstream of IGF). These scenarios would predict that the compound mutant recapitulates the *Ihh*$^{n/n}$ phenotype. However, our studies revealed that loss of both signals resulted in an additive reduction in the lengths of long bones. Therefore, these observations support an alternative conclusion that Ihh and IGF1R most likely signal independently to control skeletal growth.

The independence between Ihh and IGF may indicate distinct mechanisms utilized by the two signals in regulating skeletal growth. In particular, Ihh and IGF may control chondrocyte proliferation by different means, as BrdU pulse labeling experiments consistently revealed an ~50% reduction in the labeling index in cells lacking Ihh signaling but failed to detect a deficit in the proliferating *Igf1r*$^{n/n}$ chondrocytes. The normal BrdU labeling index indicates that the S phase was not reduced in relation to other phases of the cell cycle in the *Igf1r*$^{n/n}$ cells. However, it is possible that all phases of the cell cycle were prolonged in the *Igf1r*$^{n/n}$ chondrocytes, resulting in a longer total cell cycle time and hence a reduction in proliferation. Indeed, a previous study showed that *Igf1r*$^{n/n}$ embryonic fibroblasts in culture exhibited a cell cycle time more than twice that of normal fibroblasts, with all phases of the cell cycle prolonged and the relative portion of the S phase unchanged (Sell et al., 1994). Notably, the previous study also reported that pulse labeling experiments with [^{3}H]thymidine did not detect any difference between the wild-type and the *Igf1r*$^{n/n}$ fibroblasts in culture (Sell et al., 1994).

In addition to its role in chondrocyte proliferation, Ihh may also directly regulate chondrocyte morphology. In support of this notion, removal of Ihh responsiveness (*Smo-CKO*) resulted in a dramatic reduction in the number of flattened cells and the absence of a distinct columnar region. The regulation of cell morphology may be independent of chondrocyte maturation, as the latter appeared largely normal in these animals (Long et al., 2001). Alternatively, loss of the columnar region could be secondary to the marked decrease in cell number in the absence of Ihh signaling as cells within this region normally exhibit both the highest proliferation rate and the most robust Ihh signaling within the growth plate (Long et al., 2001). Regardless of the mechanism, this finding is consistent with a model wherein Ihh, independent of PTHrP, promotes the length of the columnar region in the growth plate (Kobayashi et al., 2005).

Our studies revealed that removal of *Igf1r* specifically in collagen-II-expressing cells (chondrocytes and osteoblast progenitors) resulted in significant growth retardation in long bones. In contrast, specific deletion of *Igf1r* in mature osteoblasts by osteocalcin-Cre resulted predominantly in mineralization defects without obvious growth deficits (Zhang et al., 2002). Thus, it appears that IGF signaling modulates skeletal growth chiefly by acting on chondrocytes and possibly osteoblast precursors.

Acknowledgments

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